Effects of isoflavone derivatives on the production of inflammatory cytokines by synovial cells

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Abstract. The present study investigated the effects of isoflavone derivatives (daidzein, genistein and glycitein) on the production of inflammatory cytokines (IL-6 and IL-8) by IL-1β-stimulated synovial cells. Synovial MH7A cells were stimulated with IL-1β in the absence or presence of isoflavone derivatives, and IL-6 and IL-8 production was measured by ELISA. The results of the present study indicated that daidzein significantly inhibited the production of IL-6, but not IL-8. Conversely, neither genistein nor glycitein exerted any inhibitory effects on the production of IL-6 or IL-8 by IL-1β-stimulated synovial cells. To elucidate the molecular mechanisms underlying the daidzein-mediated inhibition of IL-6 production, the present study examined the effects of daidzein on the phosphorylation (activation) of NF-κB p65, ERK1/2 and p38 MAPK. Daidzein significantly inhibited the phosphorylation of NF-κB p65 and ERK1/2, but not p38 MAPK in IL-1β-stimulated MH7A cells. The present study revealed that among the isoflavone derivatives examined (daidzein, genistein and glycitein), daidzein inhibited the production of IL-6, but not IL-8, by IL-1β-stimulated synovial MH7A cells via the suppression of NF-κB p65 and ERK1/2 activation. Collectively, these results suggested that daidzein may have potential as a therapeutic agent for the treatment of arthritic disorders through its anti-inflammatory effects via the inhibition of IL-6 production.

Introduction

Activated synovial cells are involved in the pathogenesis and progression of arthritic diseases, such as rheumatoid arthritis and osteoarthritis (1,2). The pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are typical activators of synovial cells (3). They stimulate their own production and also induce the production of other inflammatory cytokines and mediators, including IL-6, IL-8, nitric oxide (NO), and prostaglandin E_2, by synovial cells (4,5). Increased levels of these inflammatory cytokines and mediators induce persistent inflammation and cartilage degradation, eventually leading to joint destruction (1). Therefore, the inhibition of inflammatory cytokine and mediator production by activated synovial cells is regarded as a potential therapeutic target for suppressing the progression of inflammation and joint destruction in arthritic diseases (6).

Isoflavone derivatives (isoflavones) are a class of flavonoids that belong to a large family of polyphenolic compounds from plants (such as soybeans, red clover, and kudzu roots) (7-9). Isoflavones are also called phytoestrogens because they contain an estrogen structure (particularly 17β-estradiol) (Fig. 1), and mimic the effects of estrogens by binding to estrogen receptors (10-12). Isoflavones are divided into a glycosidic form with sugar chains and an aglycone form without sugar chains in their molecular structures (8). Daidzein, genistein, and glycitein are the aglycone forms of isoflavones (Fig. 1), and exhibit more potent bioactivities than their glycoside forms (10). In addition to estrogenic activity, isoflavones possess various biological properties (such as antioxidant, antimicrobial, and anti-inflammatory activities) (13-15). The anti-inflammatory activities of isoflavones have been examined in detail, and their therapeutic effects have been demonstrated in various animal models with inflammatory disorders, such as lung injury and arthritis (16,17). A previous study confirmed the anti-inflammatory effects of genistein in a collagen-induced rheumatoid arthritis model of rats (17). Genistein, daidzein, and glycitein also exert anti-inflammatory effects through the inhibition of inflammatory cytokine and mediator production by various types of cells, including macrophages, chondrocytes, and synovial cells (18-21). For example, genistein reduced the LPS-stimulated up-regulation of cyclooxygenase (COX)-2 and NO production in primary cultures of human chondrocytes (18). Genistein also decreased the production of IL-1β, IL-6, and IL-8 by the TNF-α-simulated fibroblast-like synovial cell line MH7A (19). Moreover, daidzein inhibited LPS-stimulated NO and IL-6 production by RAW264.7 mouse macrophage cells (20), and glycitein suppressed NO production by LPS-activated RAW264.7 cells (21).

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We have been investigating the anti-inflammatory effects of various functional food materials, such as glucosamine, citrulline, methionine, and chondroitin sulfate, on IL-6 and IL-8 production by IL-1β-stimulated synovial cells (22,23), and revealed that these materials inhibit cytokine production by suppressing signaling molecules, including nuclear factor (NF)-κB, extracellular signal regulated kinases (ERK)1/2, and p38 mitogen-activated protein kinase (MAPK). However, the anti-inflammatory effects of isoflavones (daidzein, genistein, and glycitein) on inflammatory cytokine production by IL-1β-stimulated synovial cells have not yet been examined. Therefore, the present study investigated the effects of isoflavones (daidzein, genistein, and glycitein) on the production of inflammatory cytokines (IL-6 and IL-8) by IL-1β-stimulated synovial cells. The results obtained indicated that among these isoflavones, only daidzein inhibited IL-6 production by IL-1β-stimulated synovial cells, and this effect was mediated by the suppression of NF-κB and ERK1/2 activation (phosphorylation).

Materials and methods

Reagents and antibodies. Human IL-1β (cat. no. 200-01B) was purchased from PeproTech; daidzein (cat. no. 09388-64), genistein (cat. no. NH010302), glycitein (cat. no. 09387-74), dimethyl sulfoxide (DMSO; cat. no. 09659-14), RIPA (radioimmunoprecipitation assay) buffer (50 mmol/l Tris-HCl buffer pH 7.6, 150 mmol/l NaCl, 1% Nonidet p40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), Protease Inhibitor Cocktail; cat. no. 08714-04), 2X SDS-PAGE (polyacrylamide gel electrophoresis) sample buffer (cat. no. 30657-12), 6X SDS-PAGE sample buffer (cat. no. 09500-64), and WB (western blot) Stripping Solution Strong (cat. no. 05677-65) were from Nacalai Tesque, Inc. A rabbit anti-phosphorylated NF-κB p65 polyclonal antibody (93H1; cat. no. 3033), rabbit anti-phosphorylated ERK1/2 polyclonal antibody (Thr202/Tyr204; cat. no. 9101), rabbit anti-NF-κB p65 (D14E12; cat. no. 8242), and rabbit anti-ERK1/2 (cat. no. 9102) were purchased from Cell Signaling Technology, Inc.; a mouse anti-phosphorylated p38 MAPK monoclonal antibody (clone 30; cat. no. 612281) and mouse anti-p38 MAPK (clone 27; cat. no. 612168) from BD Bioscience Pharmingen; a mouse anti-GAPDH monoclonal antibody (cat. no. MAB374) from Chemicon International; and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin IgG (cat. no. 115-035-144) and HRP-conjugated goat anti-mouse IgG/IgM (cat. no. 115-035-044) from Jackson ImmunoResearch Laboratories, Inc.

Daidzein, genistein, and glycitein were dissolved in DMSO immediately before use, sterilized by filtration, and then used in experiments.

Cell culture. The human fibroblast-like synovial cell line MH7A (RCB1512) obtained from the Riken Cell Bank was cultured in RPMI-1640 medium (Nacalai Tesque, Inc.) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque, Inc.) at 37˚C in a humidified incubator containing 5% CO_2, as previously described (24).

Measurement of IL-6 and IL-8. MH7A cells were seeded on 24-well culture plates at a density of $3.5x10^4$ cells per well and incubated overnight in RPMI-1640 medium containing 10% FBS. Cells were then pretreated without or with daidzein (1 or 5 μg/ml), genistein (1 or 5 μg/ml), and glycitein (1 or 5 μg/ml) for 3 h, and stimulated at 37˚C for 18 h with or without 50 pg/ml IL-1β in a total volume of 0.5 ml RPMI-1640 medium. Thereafter, culture supernatants were collected, centrifuged at 3,600 x g at 4˚C for 5 min, and the concentrations of IL-6 and IL-8 in the culture supernatants were assessed by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc.) according to the manufacturer's instructions. Briefly, 96-well half area plates were coated with 0.6 μg/ml of the mouse anti-human IL-6 antibody or 0.7 μg/ml of the mouse anti-human IL-8 antibody diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; pH 7.4) and incubated at 4˚C overnight. The plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS containing 1% bovine serum albumin at room temperature for 1 h. Thereafter, plates were washed three times with PBS-T and incubated with culture supernatants or standards (5-1,000 pg/ml) at room temperature for 2 h. After washing three times with PBS-T, plates were incubated with 15 ng/ml of the biotinylated goat anti-human IL-6 antibody or 7 ng/ml of the biotinylated goat anti-human IL-8 antibody at room temperature for 2 h, washed three times with PBS-T, and then incubated with streptavidin-HRP at room temperature for 30 min. Plates were then washed five times with PBS-T and incubated with tetramethyl benzidine (TMB) liquid substrate at room temperature in the dark until color developed. The reaction was terminated using 1 M H_2SO_4 and the absorbance of the solution was measured with a xMARK™ Microplate Spectrophotometer (Model 680; Bio-Rad Laboratories, Inc.) at 450 nm.

Phosphorylation of NF-κB, ERK1/2, and p38 MAPK. MH7A cells ($1.5x10^5$/well) were seeded and cultured in 6-well plates overnight. Cells were then pretreated without or with 10 μg/ml daidzein for 16 h and stimulated with 200 pg/ml IL-1β at 37˚C for 15 or 30 min. Cells were washed twice with ice-cold PBS, and then lysed in 150 μl/well RIPA buffer containing Phosphatase Inhibitor Cocktail (Roche Diagnostics; cat. no. 04 906 837 001). Following sonication, lysates were centrifuged at 15,000 x g at 4˚C for 15 min and the supernatants were collected. The protein concentrations of the supernatants were assessed using the Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology), and 12 μg protein/lane was used in the western blot analysis. Proteins were separated by 10% SDS-PAGE and transferred onto an PVDF (polyvinylidene difluoride) membrane (EMD Millipore), followed by blocking with Block Ace solution (Megmilk Snow Brand Co., Ltd.) for 1 h. Membranes were then washed three times with Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.05% Tween-20 (TBS-T), and incubated with the rabbit anti-phosphorylated NF-κB p65 polyclonal antibody (1,000-fold dilution), rabbit anti-phosphorylated ERK1/2 polyclonal antibody (1,000-fold dilution), or mouse anti-phosphorylated p38 MAPK monoclonal antibody

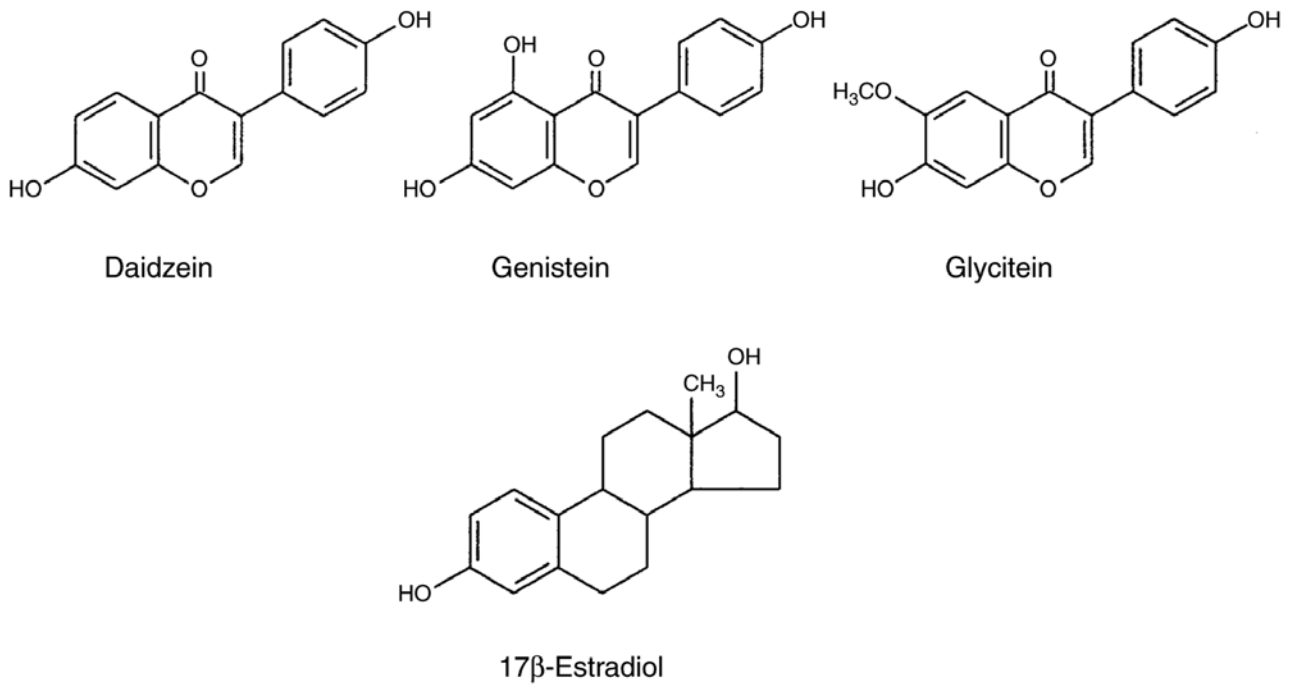

Figure 1. Structures of isoflavones (daidzein, genistein, and glycitein) and 17β-estradiol.

(1,000-fold dilution) in TBS containing 0.05% NaN_3 at 4˚C overnight. After washing three times with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (5,000-fold dilution) or HRP-conjugated goat anti-mouse IgG/IgM (5,000-fold dilution) in TBS containing 10% Block Ace solution for 1 h. Following washing three times with TBS-T, reactive proteins were detected with SuperSignal™ West Dura Extended Duration Substrate (Pierce Biotechnology), and signals were quantified using the LAS-3000 luminescent image analyzer (Fujifilm Corporation) and Multi Gauge version 3.0 (Fujifilm Corporation) with the Exposure type of Increment and the View menu of Paint saturated data Red, in which images were analyzed before the saturation of the images.

Membranes were then stripped with WB Stripping Solution Strong for 30 min and reprobed with rabbit anti-NF-κB p65 (5,000-fold dilution), rabbit anti-ERK1/2 antibodies (1,000-fold dilution), or mouse anti-p38 MAPK (2,000-fold dilution) followed by an incubation with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG/IgM secondary antibodies, respectively.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with a mouse anti-GAPDH monoclonal antibody (50,000-fold dilution; cat. no. MAB374; Chemicon International) and HRP-conjugated goat anti-mouse IgG/IgM (5,000-fold dilution).

Reactive proteins were detected and their signals were quantified, as described above.

Statistical analysis. Data were expressed as the mean ± standard deviation, and analyzed for significant differences by a one-way ANOVA with a post-hoc Tukey's test using GraphPad Prism software version 6.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a significant difference.

Results

Effects of daidzein, genistein, and glycitein on IL-6 and IL-8 production by IL-1β-stimulated MH7A cells. Fig. 2 shows the effects of daidzein, genistein, and glycitein on IL-6 and IL-8 production by MH7A cells. Cells were stimulated with IL-1β in the absence or presence of daidzein (1 or 5 μg/ml), genistein (1 or 5 μg/ml), or glycitein (1 or 5 μg/ml) for 18 h, and the levels of IL-6 and IL-8 were measured by ELISA. The production of IL-6 (Fig. 2A-C) was approximately 28-fold higher after the IL-1β stimulation (1,090±479 pg/ml) than that by unstimulated cells (38±35 pg/ml). Daidzein (5 μg/ml) markedly inhibited the production of IL-6 by approximately 20% from that by the IL-1β stimulation ($P=0.3$ compared with IL-1β stimulation + DMSO, and $P=0.1$ compared with IL-1β stimulation only), whereas 1 μg/ml daidzein did not significantly affect IL-6 production (Fig. 2A). On the other hand, genistein and glycitein did not exert any significant effects on IL-6 production in IL-1β-stimulated MH7A cells (Fig. 2B and C). Furthermore, the production of IL-8 was approximately 127-fold higher with the IL-1β stimulation (2,359±1,206 pg/ml) than that by unstimulated cells (19±33 pg/ml). However, neither daidzein, genistein, nor glycitein exerted any significant effects on IL-8 production (Fig. 2D-F).

A morphological analysis of the cytotoxic effects of daidzein on IL-1β-stimulated human synovial MH7A cells was performed under light microscopy. Daidzein did not induce cell death (such as apoptosis and necrosis) in IL-1β-stimulated MH7A cells during an incubation at 5 and 10 μg/ml (data not shown). Consistent with this result, similar amounts of GAPDH, an internal control, were recovered from the cell lysates of unstimulated MH7A cells, IL-1β-stimulated MH7A cells, and IL-1β-stimulated MH7A cells incubated with daidzein (Figs. 3 and 4).

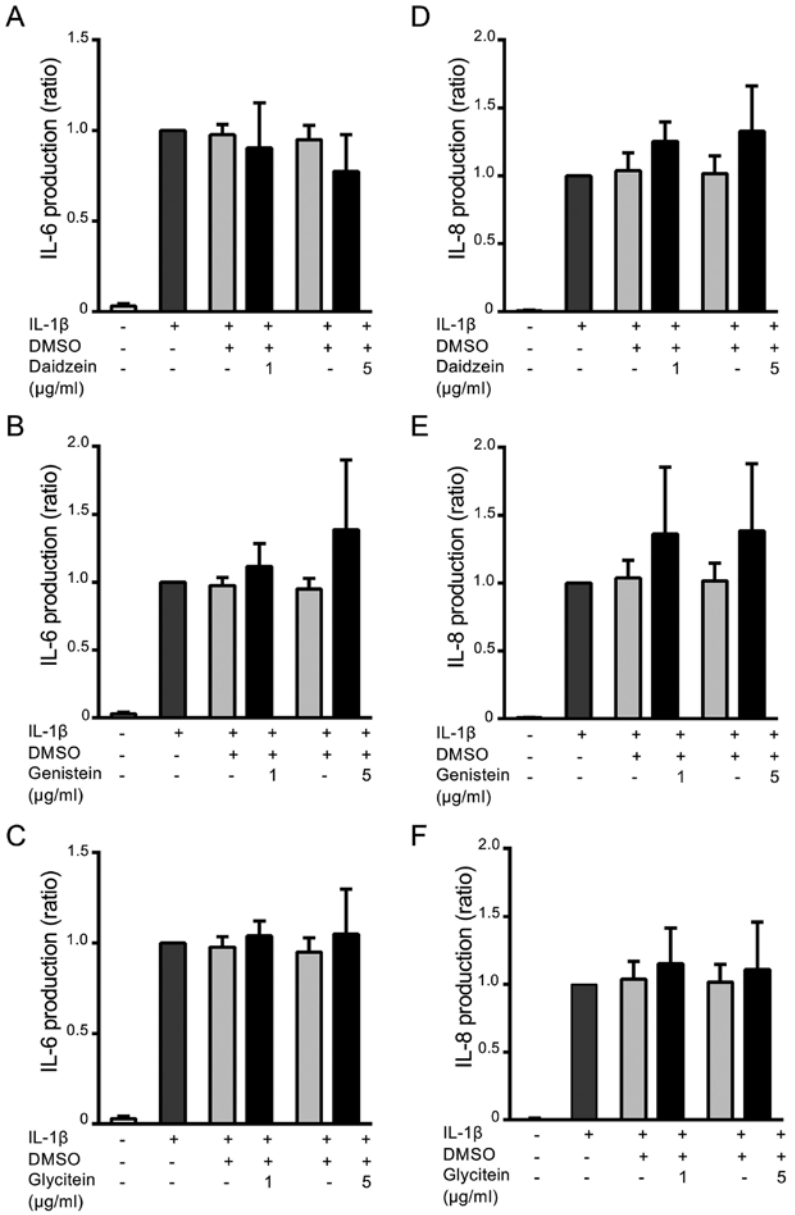

Figure 2. Effects of daidzein, genistein, and glycitein on IL-6 and IL-8 production by IL-1β-stimulated MH7A cells. Cells were treated without (-) or with (+) 1 or 5 μg/ml of (A and D) daidzein, (B and E) genistein or (C and F) glycitein for 3 h, and then stimulated without (-) or with (+) 50 pg/ml IL-1β for 18 h. Alternatively, cells were treated without (-) or with (+) 0.5% DMSO (a solvent of isoflavones) in the absence of isoflavones, and then stimulated with IL-1β. Thereafter, culture supernatants were recovered, and (A-C) IL-6 and (D-F) IL-8 levels were measured by ELISA. Data are expressed as a ratio to that in IL-1β-stimulated MH7A cells without isoflavone derivatives, and compared between without and with isoflavones. Results are shown as the means ± SD of five independent experiments. DMSO, dimethyl sulfoxide.

Effects of daidzein on the phosphorylation of NF-κB, ERK1/2, and p38 MAPK. The activation of the NF-κB and MAPK (ERK1/2 and p38 MAPK) signaling pathways is involved in the production of inflammatory cytokines. Therefore, to elucidate the mechanisms underlying the daidzein-mediated suppression of IL-6 production, we examined the activation (phosphorylation) of NF-κB p65, ERK1/2, and p38 MAPK. MH7A cells were stimulated with 200 pg/ml IL-1β for 15 or 30 min after the treatment with or without 10 μg/ml daidzein for 16 h. The phosphorylation of NF-κB p65, ERK1/2, and p38 MAPK was evaluated by western blotting. When cells were stimulated with IL-1β for 15 min, the phosphorylation of NF-κB p65 was approximately 17-fold higher (Fig. 3A) than that in unstimulated cells. Daidzein (10 μg/ml) significantly inhibited the phosphorylation of NF-κB p65 by 23% from that by the IL-1β stimulation only (Fig. 3A). The phosphorylation of ERK1/2 and p38 MAPK was also increased by approximately 1.7- and 5-fold, respectively, by the IL-1β stimulation (Fig. 3B and C). However, daidzein (10 μg/ml) did not show exert any significant effects on the phosphorylation of ERK1/2 or p38 MAPK (Fig. 3B and C).

We also examined the effects of daidzein (10 μg/ml) on the phosphorylation of NF-κB p65, ERK1/2, and p38 MAPK after the stimulation with IL-1β for 30 min. After the stimulation, the phosphorylation of NF-κB p65, ERK1/2, and p38 MAPK was approximately 23-, 2-, and 2.6-fold higher, respectively, that that in unstimulated cells (Fig. 4). Daidzein (10 μg/ml) significantly inhibited the IL-1β-induced phosphorylation

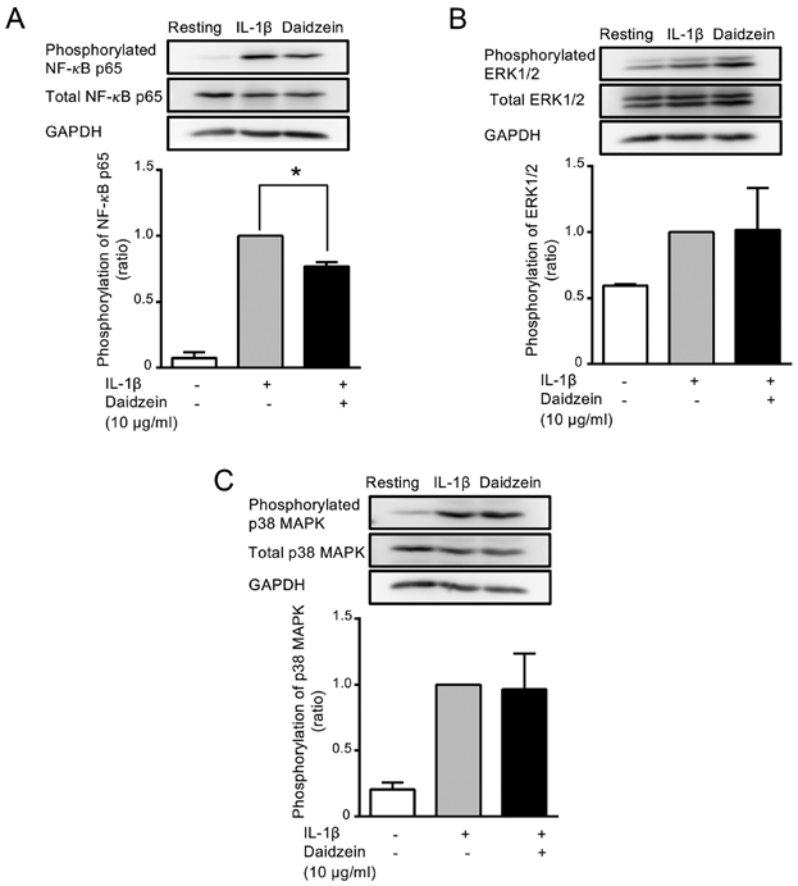

Figure 3. Effects of daidzein on the phosphorylation of NF-κB, ERK1/2, and p38 MAPK. Cells were treated without (-) or with (+) 10 μg/ml daidzein for 16 h, and then stimulated without (-) or with (+) 200 pg/ml IL-1β for 15 min. Cells were harvested, the supernatants (containing 12 μg protein) obtained from cell lysates were resolved on 10% SDS-PAGE, and phosphorylated and total (A) NF-κB p65, (B) ERK1/2 and (C) p38 MAPK were evaluated by western blotting. GAPDH (an internal control) was also detected by western blotting. Images are representative of three separate experiments. The phosphorylation of NF-κB p65, ERK1/2, and p38 MAPK was normalized with total NF-κB p65, ERK1/2, and p38 MAPK, respectively, and expressed as a ratio to that in IL-1β-stimulated MH7A cells without daidzein. Data were compared between without and with daidzein. Results are expressed as the means ± SD of three independent experiments. *P<0.05. NF-κB, nuclear factor-κB; ERK, extracellular signal regulated kinases; p38 MAPK, p38 mitogen-activated protein kinase.

of ERK1/2 by 50% (Fig. 4B). In contrast, daidzein did not exert any significant effects on the phosphorylation of p65 or p38 MAPK after the 30-min stimulation (Fig. 4A and C).

Therefore, daidzein inhibited the IL-1β-induced phosphorylation of NF-κB p65 (after 15 min) and ERK1/2 (after 30 min) in MH7A cells.

Discussion

The present study investigated the effects of isoflavone derivatives (daidzein, genistein, and glycitein) on the production of inflammatory cytokines (IL-6 and IL-8) by IL-1β-stimulated synovial MH7A cells. The results obtained indicated that daidzein markedly inhibited the production of IL-6, but not IL-8. In contrast, neither genistein nor glycitein exerted any effects on IL-6 or IL-8 production by IL-1β-stimulated synovial cells.

A previous study reported that genistein significantly inhibited the production of IL-6 and IL-8 by TNF-α-stimulated synovial MH7A cells (19). However, in the present study, genistein did not inhibit the production of IL-6 or IL-8 by IL-1β-stimulated synovial MH7A cells. Therefore, genistein may exert different effects on cytokine production depending on the stimuli used (different stimuli of IL-1β and TNF-α). In addition, the present study indicated that glycitein did not affect IL-6 or IL-8 production by IL-1β-stimulated synovial cells. To the best of our knowledge, the effects of glycitein on cytokine production have not yet been examined.

The signal transduction pathways of NF-κB and MAPK (ERK1/2 and p38 MAPK) play a critical role in the production of inflammatory cytokines and mediators (25-27). Therefore, to elucidate the molecular mechanisms underlying the daidzein-mediated inhibition of IL-6 production, we examined the effects of daidzein on the phosphorylation (activation) of NF-κB p65, ERK1/2, and p38 MAPK. The results obtained indicated that daidzein significantly inhibited the phosphorylation of NF-κB p65 (15 min) and ERK1/2 (30 min) by MH7A cells after the IL-1β stimulation. In contrast, daidzein did not exert any effects on the phosphorylation of p38 MAPK. A previous study reported that daidzein inhibited the phosphorylation of NF-κB p65 in lipopolysaccharide (LPS)-stimulated murine J774 macrophages (28). However, the effects of daidzein on the phosphorylation of ERK1/2 currently remain unknown. Following the IL-1β stimulation, TAK1 (transforming growth factor-β-activated kinase 1; an isoform of MAP kinase kinase kinase; MAPKKK) was shown to be activated, and activated TAK1 phosphorylated the inhibitor of κB (IκB) kinase (IKK) (Fig. 5) (4,29,30). Activated IKK phosphorylates IκB in the

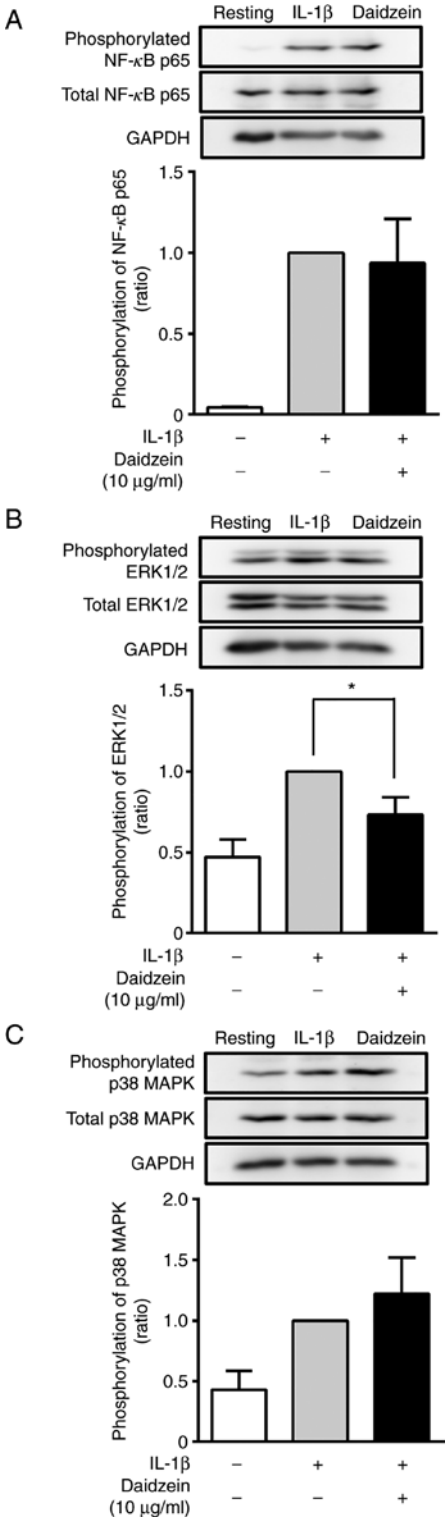

Figure 4. Effects of daidzein on the phosphorylation of NF-κB, ERK1/2, and p38 MAPK. Cells were treated without (-) or with (+) 10 μg/ml daidzein for 16 h and then stimulated without (-) or with (+) 200 pg/ml IL-1β for 30 min. Thereafter, cells were harvested, the supernatants (containing 12 μg protein) obtained from cell lysates were resolved on 10% SDS-PAGE, and phosphorylated and total (A) NF-κB p65, (B) ERK1/2 and (C) p38 MAPK were evaluated by western blotting. GAPDH (an internal control) was also detected by western blotting. Images are representative of three separate experiments. The phosphorylation of NF-κB p65, ERK1/2, and p38 MAPK was normalized to total NF-κB p65, ERK1/2, and p38 MAPK, respectively, and expressed as a ratio to that in IL-1β-stimulated MH7A cells without daidzein. Data are compared between without and with daidzein. Results are expressed as the means ± SD of three independent experiments. *P<0.05. NF-κB, nuclear factor-κB; ERK, extracellular signal regulated kinases; p38 MAPK, p38 mitogen-activated protein kinase.

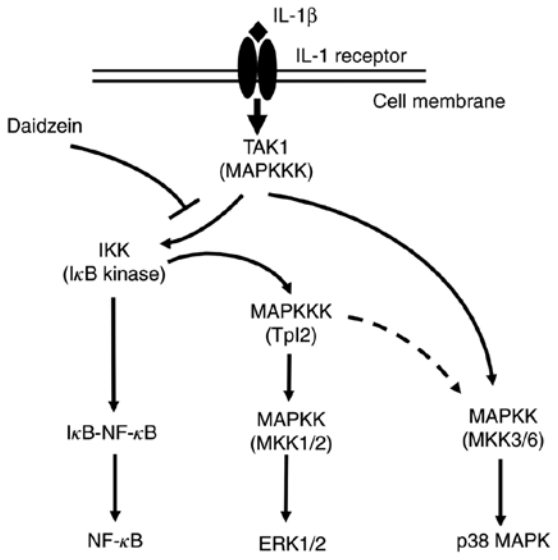

Figure 5. Schematic model of IL-1β-stimulated signaling pathways of NF-κB p65, ERK1/2, and p38 MAPK, and inhibitory effects of daidzein on NF-κB/ERK1/2 signaling in synovial cells. TAK1 is activated by a stimulation with IL-1β, and activated TAK1 phosphorylates the IKK. Activated IKK phosphorylates IκB in the complex with NF-κB, and phosphorylated IκB is degraded by the ubiquitin-proteasome system, which allows the release and nuclear translocation of NF-κB, leading to the transcription of inflammatory cytokine genes. Moreover, activated IKK activates Tpl2, which, in turn, activates MKK1/2, leading to the activation of ERK1/2. Alternatively, TAK1 activates the p38 MAPK pathway via the activation of MKK3 and MKK6. p38 MAPK may also be activated by TAK1 via the IKK/Tpl2 pathway (dotted line). Since daidzein inhibited the phosphorylation of NF-κB p65 and ERK1/2, but not p38MAPK in IL-1β-stimulated MH7A cells, it appears to inhibit the TAK1/IKK-mediated activation of NF-κB p65 and ERK1/2, but not the TAK1/MKK3/MKK6-mediated activation of p38 MAPK. IKK, IκB kinase; IκB, inhibitor of κB. NF-κB, nuclear factor-κB; ERK, extracellular signal regulated kinases; p38 MAPK, p38 mitogen-activated protein kinase; TAK1, transforming growth factor-β-activated kinase 1; MAPKKK, MAP kinase kinase kinase; IκB, inhibitor of κB; IKK, IκB kinase; Tpl2, tumor progression locus 2; MKK and MAPKK, mitogen-activated protein kinase kinase.

complex with NF-κB, and phosphorylated IκB is degraded by the ubiquitin-proteasome system, which allows the release and nuclear translocation of NF-κB, leading to the transcription of inflammatory cytokine genes (Fig. 5) (29-32). Moreover, activated IKK activates Tpl2 (tumor progression locus 2; an isoform of MAPKKK), which, in turn, activates MKK (mitogen-activated protein kinase kinase; MAPKK) 1/2, leading to the activation of ERK1/2 (Fig. 5) (33). Alternatively, TAK1 activates the p38 MAPK pathway via the activation of MKK3 and MKK6 (33). p38 MAPK may also be activated by TAK1 via the IKK/Tpl2 pathway (33). The present study revealed that daidzein inhibited the phosphorylation of NF-κB p65 and ERK1/2 (Figs. 3A and 4B), but not p38MAPK in IL-1β-stimulated MH7A cells. Therefore, it is reasonable to speculate that daidzein primarily inhibits the TAK1/IKK-mediated activation of NF-κB p65 and ERK1/2, but not the TAK1/MKK3/MKK6-mediated activation of p38 MAPK. Moreover, daidzein significantly inhibited the phosphorylation of NF-κB p65 (15 min) and ERK1/2 (30 min) by MH7A cells after the IL-1β stimulation. The time difference in the inhibitory effects of daidzein on the phosphorylation of NF-κB p65 (15 min) and ERK1/2 (30 min) may reflect differences in the activation of NF-κB p65 (activation at an early

stage) and ERK1/2 (activation at a later stage) via the different signal transduction pathways starting from TAK1 by the IL-1β stimulation in MH7A cells.

NF-κB is a pivotal transcription factor for the expression of inflammatory cytokines, such as IL-6 and IL-8 (25,34). After its stimulation, NF-κB translocates to the nucleus and binds to the NF-κB binding site of the promoters for cytokine genes, leading to cytokine gene expression (35-37). In the present study, daidzein significantly inhibited the phosphorylation of NF-κB p65 (Fig. 3A) and production of IL-6 (Fig. 2A), but not IL-8 (Fig. 2D). IL-6 production was approximately 28-fold higher after the IL-1β stimulation than that by unstimulated cells, whereas IL-8 production was increased by approximately 127-fold by the IL-1β stimulation. Daidzein inhibited the phosphorylation of NF-κB p65 by only 20% (Fig. 3A). Therefore, the expression of IL-6 appears to be more sensitive to the inhibitory effects of daidzein than that of IL-8, and the weak daidzein-induced inhibition of NF-κB p65 phosphorylation (approximately 20%) may suppress the moderate elevations in IL-6 expression, but not the marked increases in IL-8 expression. However, further studies are needed to clarify the mechanisms responsible for the different effects of daidzein on IL-6 and IL-8 production.

In the present study, the concentrations of daidzein (5 and 10 μg/ml) and the incubation periods with daidzein (3 and 16 h) are different between ELISA (detection of cytokines IL-6 and IL-8) and western blot analysis (detection of the phosphorylation of NF-κB, ERK1/2 and p38 MAPK). This is based on the facts that the detection of cytokines by ELISA is highly sensitive. Thus, cytokine production was markedly increased by a low concentration of IL-1β (50 pg/ml), and especially IL-6 production was significantly suppressed by incubation with 5 μg/ml daidzein for 3 h (as shown in Fig. 2A). In contrast, the detection of the phosphorylation of NF-κB, ERK1/2 and p38 MAPK was low-sensitive, and the phosphorylation was significantly enhanced by a high concentration of IL-1β (200 pg/ml). Thus, to make the suppressive effect of daidzein more effective, MH7A cells were incubated with a high concentration of daidzein (10 μg/ml) for a long period (16 h) to detect the effect of daidzein on the phosphorylation of NF-κB, ERK1/2 and p38 MAPK (Figs. 3 and 4), compared with the effect of daidzein on cytokine production (5 μg/ml daidzein and incubation period 3 h) (Fig. 2).

In conclusion, the present study revealed that among the isoflavone derivatives examined (daidzein, genistein, and glycitein), daidzein inhibited the production of IL-6, but not IL-8 by IL-1β-stimulated synovial MH7A cells, possibly via the suppression of NF-κB p65 and ERK1/2 activation. IL-6 is a pleiotropic cytokine that plays a pivotal role in the pathophysiology of arthritis, which is found in the synovial fluid, and its level correlates with disease activity and joint destruction (38). Furthermore, IL-6 may promote synovitis and joint destruction by stimulating neutrophil migration, osteoclast maturation, and vascular endothelial growth factor (VEGF)-stimulated pannus proliferation (38). In contrast, IL-8 is an inflammatory chemokine that is involved in the pathological processes of arthritis, including the release of matrix metalloproteinase-13 (MMP-13), neutrophil accumulation, and leukocyte homing to the synovium (39). Based on these findings, daidzein has potential as a therapeutic agent for arthritic disorders by mainly suppressing IL-6 production, thereby ameliorating the progression of inflammation and joint destruction.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NM substantially contributed to the conception and design of this study, and the acquisition of data. AS and IN confirmed the authenticity of all the raw data. NM, AS and IN were involved in the analysis and interpretation of data, and drafting and revising the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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